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New *Bradyrhizobium japonicum* Strains That Possess High Copy Numbers of the Repeated Sequence RSα

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In a survey of DNA fingerprints of indigenous *Bradyrhizobium japonicum* with the species-specific repeated sequences RSα and RSβ, 21 isolates from three field sites showed numerous RS-specific hybridization bands. The isolates were designated highly reiterated sequence-possessing (HRS) isolates, and their DNA hybridization profiles were easily distinguished from the normal patterns. Some HRS isolates from two field sites possessed extremely high numbers of RSα copies, ranging from 86 to 175 (average, 128), and showed shifts and duplications of *nif*- and *hup*-specific hybridization bands. The HRS isolates exhibited slower growth than normal isolates, although no difference in symbiotic properties was detected between the HRS and normal isolates. Nucleotide sequence analysis of 16S rRNA genes showed that HRS isolates were strains of *B. japonicum*. There was no difference in the spectra of serological and hydrogenase groupings of normal and HRS isolates. Some HRS isolates possessed a tandem repeat RSα dimer that is similar to the structure of $(IS30)_2$, which was shown to cause a burst of transpositional rearrangements in *Escherichia coli*. The results suggest that HRS isolates are derived from normal isolates in individual fields by genome rearrangements that may be mediated by insertion sequences such as RSα.

Insertion sequence (IS) elements are discrete segments of DNA that are able to transpose to numerous sites on bacterial plasmids and chromosomes, usually with an increase in their copy number (7). IS elements can also promote rearrangement of genomes and other replicons (7). Many IS elements and uncharacterized repeated DNA sequences among plant-associated gram-negative bacteria, including *Agrobacterium* (5), *Bradyrhizobium* (10, 11, 17), *Rhizobium* (3, 5, 35), and *Xanthomonas* (2) spp., have been described. These repeated elements often cause genomic instability affecting genes responsible for plant associations (15, 23), and they have been postulated to play a role in evolution and genomic instability (1, 7, 17, 24, 29). Indeed, complete sequencing of a symbiotic plasmid pNGR234a from a *Rhizobium* sp. demonstrated that almost one-fifth of the total plasmid sequence is made up of IS elements and a mosaic sequence structure including nodulation loci (6).

Members of the genus *Bradyrhizobium* are slow-growing, gram-negative, nitrogen-fixing heterotrophic bacteria which can form root nodules on several leguminous plants. In *Bradyrhizobium japonicum*, several repeated DNA sequences (RSα, RSβ, RSγ, RSδ, RSε, and RSζ) have been identified (10, 11, 17). At least one of these sequences, RSα, has structural properties similar to that of a prokaryotic IS element. Interestingly, the RS copies are often clustered around the regions of nitrogen-fixation and nodulation genes on the chromosome of *B. japonicum* USDA110 (17). An insertion sequence, HRS1, also was found to be closely linked to common and genotype-specific nodulation genes in *B. japonicum* serocluster USDA123 and USDA127 strains (15, 27).

DNA fingerprints with RSα, RSβ, and HRS1 as probes revealed genetic diversity within natural populations of *B. japonicum* that nodulated soybeans (13, 15, 22, 27), indicating that RS fingerprinting is useful for isolate or strain identification and is a valuable tool for evaluating the genetic structure of indigenous *B. japonicum* populations.

In a previous paper (22) two *B. japonicum* isolates, NC32a and NC3a, obtained from a Nakazawa field site showed numerous bands of RS-specific hybridization. Of 213 isolates of soybean bradyrhizobia indigenous to six field sites in Japan (reference 22 and unpublished data), 19 isolates have been found to exhibit numerous bands of RS-specific hybridization, suggesting that the distribution of such isolates is ubiquitous. In this study, we have genetically and phenotypically characterized field isolates of soybean bradyrhizobia showing numerous bands of RS-specific hybridization as first steps toward gaining some understanding of their ecological role.

MATERIALS AND METHODS

Isolation of *B. japonicum* from fields. Seeds of soybean (*Glycine max*) cultivar Enrei were surface sterilized by immersion in 0.5% sodium hypochlorite for 5 min followed by washings with sterile water. Seeds were sown in sterile vermiculite and then inoculated with a moist soil sample (1 g), which had been collected from the plow layers of the Tokachi field at Tokachi Agricultural Station (Memuro, Tokachi, Hokkaido, Japan), the Nakazawa and Nagakura fields at Niigata Agricultural Experiment Station (Nagaoka, Niigata, Japan), the Ami field at the experimental farm of Ibaraki University (Ami, Ibaraki, Japan), the Fukuyama field at the Experimental Farm of Hiroshima University (Fukuyama, Hiroshima, Japan), or the Ishigaki field at the experimental field of Ishigaki Island Branch of Tropical Agriculture Research Center (Ishigaki, Okinawa, Japan). The inoculation procedure was as described previously (22). The plants were cultivated in a greenhouse, and nitrogen-free nutrient solution (20) was repeatedly supplied.

Nodules, which were randomly excised from host plants at 40 days after germination, were rinsed and then surface sterilized in an acidic mercuric chloride solution (0.1%, wt/vol) for 5 min (32). An inoculation needle was inserted into the cut surface of the nodule, and the cells adhering to the needle were streaked onto yeast extract-mannitol (YM) agar plates (20, 21).

Bacterial strains and media. *B. japonicum* strains USDA110, USDA122, and USDA123, obtained from H. H. Keyser of the U.S. Department of Agriculture, Beltsville, Md., were used as standard strains. A total of 213 soybean bradyrhizobia were isolated from the six field sites described above, but the data shown in this work were mainly obtained from three sites, i.e., Tokachi, Nakazawa, and Nagakura, for which the prefixes T, NC, and NK were used for field isolates, respectively. *B. japonicum* strains were grown aerobically at 30°C in HM salt medium (4) supplemented with 0.1% arabinose–0.025% yeast extract (Difco),

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referred to here as HM medium, and in YM medium (20, 21). *Escherichia coli* HB101 (*recA*$^-$ *hsdR hsdM pro leu* Str^r) was grown in Luria-Bertani medium (19).

DNA isolation and hybridization. Total DNA isolation and hybridization were carried out as described previously (21). RSα- and RSβ-specific probes and *hup* probe were prepared from pRJ676 (14) and pHU52 (31), respectively, as described previously (22).

Estimation of copy numbers of RSα and RSβ. Each lane of nylon membrane hybridized with ^{32}P-labeled probes was cut with a razor blade. The strip was placed into a scintillation vial containing 10 ml of 0.4 M NaOH solution and then incubated at 45°C for 30 min to dissolve the ^{32}P-labeled probe in the solution. The radioactivity of ^{32}P was measured by Cerenkov counting in a Beckman liquid scintillation counter (model LS6500). To estimate the copy numbers of RSα and RSβ, the radioactivity of each isolate was compared to that of USDA110, which possesses 12 and 6 copies of RSα and RSβ, respectively (17). Each result is a mean of duplicate determinations.

Determination of mean generation time. Precultures (0.5 ml) of *B. japonicum* strains and the field isolates at a mid-log stage were inoculated into 100 ml of HM medium. The turbidity (A_{660}) of cultures grown aerobically at 30°C was measured every 6 h with a Shimadzu spectrophotometer (model UV-1200). The mean generation time was calculated from the maximum growth rate at an early exponential phase of duplicate cultures.

Determinations of serological and symbiotic phenotypes. For serotype determination, each isolate was tested for agglutination reactions as previously described (22). Surface-sterilized soybean seeds (*Glycine max* cv. Enrei) were inoculated with a culture of each isolate grown in YM medium. Plants were cultivated as described above. About 40 days after germination, the plants were subjected to analyses for nodulation, nitrogen fixation, and hydrogenase activity. The number and fresh weight of nodules excised from the roots were measured. To measure nitrogen-fixing activity, acetylene reduction was assayed by using the nodulated roots over a period of 15 min as described by Hardy et al. (12). Hydrogenase activity was determined amperometrically with homogenates of soybean nodules as previously described (22).

16S rRNA gene sequencing. Primers 27fR (5′-CAGGAAACAGCTATGACC AGAGTTTGATCCTGGCTCAG-3′) and 1069rU (5′-TGTAAAACGACGGC CAGTCCAACATTCACACACGAG-3′) were used for 16S rRNA gene sequencing. The sequences correspond to positions 8 to 27 and 1069 to 1086 in *E. coli* numbering, respectively (18), and possess the sequences of M13 reverse and universal sequencing primers at the 5′ ends, respectively. Each 50-μl reaction mixture contained 6.5 ng of total DNA of each isolate; 1.25 U of ExTaq polymerase (Takara Shuzo Co., Ltd.); 200 μM concentrations each of dATP, dCTP, dGTP, and dTTP; and 800 nM concentrations of each primer in a buffer recommended by the manufacturer. The temperature program was 30 s at 94°C and then 25 cycles of 40 s at 94°C, 40 s at 54°C, and 60 s at 72°C. After the PCR products were purified with the QIA quick PCR purification kit (QIAGEN, Inc.), direct sequencing was performed by using the PCR products as templates with a model 373A DNA sequencer and *Taq* dye primer cycle sequencing kits for 21M13 and M13Rev. (Applied Biosystems Co.). Sequences similar to those determined for 16S rRNA genes were identified in DNA databases (DDBJ/EMBL/GenBank). Based on these sequences, a phylogenetic tree was constructed by the neighbor-joining method with Clustal W (28).

Cloning and DNA sequencing of the PCR-amplified portion of the RSα tandem repeat. Four oligonucleotide primers, i.e., primers 13 (5′-CGACAACCTC AACACCCATA-3′), 14 (5′-CTTCGTATAGATCGGCTGCT-3′), 17 (5′-ACG CATACAACGACAGAGCC-3′), and 18 (5′-TCAAATCGCGCTGCAACGT C-3′), were designed for PCR amplification of the tandem repeat of RSα on the basis of the published nucleotide sequence of RSα (17). Each 100-μl reaction mixture contained 65 ng of total DNA or PCR-amplified DNA fragment; 2.5 U of Ex Taq polymerase; 200 μM concentrations each of dATP, dCTP, dGTP, and dTTP; and 400 nM concentrations of each primer in a buffer recommended by the manufacturer. The temperature program was 1 cycle of 240 s at 95°C, 60 s at 55°C, and 60 s at 72°C; 30 cycles of 60 s at 94°C, 60 s at 55°C, and 60 s 72°C; and 1 cycle of 300 s at 72°C. PCR products were separated by horizontal electrophoresis on 2% agarose gels, then stained with ethidium bromide and photographed. Products were also cloned into pCR^{TM}II with a TA cloning kit (Invitrogen Co.) according to the manufacturer's instructions. DNA sequence analysis was performed with an A.L.F. DNA sequencer II (Pharmacia) on both strands by using the M13 universal and M13 reverse primers.

Nucleotide sequence accession numbers. The nucleotide sequence data reported here will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers: AB004083 for RSα tandem repeat of HRS isolate NC32a, AB004084 for RSα tandem repeat of HRS isolate NK5, AB004085 for RSα tandem repeat of HRS isolate T2, AB004086 for RSα tandem repeat with 35-bp spacer of RSα tandem repeat in HRS isolate NK5, AB004807 for 16S rRNA gene sequence of HRS isolate NK5, AB004808 for 16S rRNA gene sequence of HRS isolate NK6, and AB004806 for 16S rRNA gene sequence of normal isolate NK2.

RESULTS

***B. japonicum* isolates possess a high copy number of RSα and RSβ.** In addition to the two *B. japonicum* isolates, NC32a and NC3a, obtained from a Nakazawa field site described in a previous paper (22), the DNA samples from 19 additional isolates from Nagakura and Tokachi field sites were found to show numerous bands of RS-specific hybridization, which were easily distinguished from normal hybridization patterns exhibited by USDA110 and USDA122. These isolates are designated highly reiterated sequence-possessing (HRS) isolates.

In order to estimate the copy number of the RS elements, the radioactivity of hybridization signals of each HRS isolate was compared with that of normal *B. japonicum* strain USDA110, which possesses 12 copies of RSα and 6 copies of RSβ (17). This analysis was based on the assumption that HRS and normal isolates do not differ in their genome sizes and their intensities of hybridization. The estimated copy numbers of RSα and RSβ in HRS isolates were much higher than those of the normal isolates (Table 1). According to the copy numbers of RS elements, HRS isolates tested were categorized into two types, the Niigata type and the Tokachi type (Table 1). Niigata-type HRS isolates from Nakazawa and Nagakura field sites possessed a significantly higher number of RSα copies than those of Tokachi- type HRS and normal isolates. The mean estimated copy numbers with standard deviations were 128 ± 25 (range, 86 to 175) for the RSα copy and 33 ± 9 (range, 22 to 45) for the RSβ copy in the Niigata-type HRS isolates and 21 ± 3 (range, 17 to 23) for the RSα copy and 44 ± 6 (range, 35 to 51) for the RSβ copy in the Tokachi-type HRS isolates. On the other hand, the copy number of normal isolates from the three fields showed 7 ± 1 (range, 5 to 9) for the RSα copy and 6 ± 3 (range, 2 to 9) for the RSβ copy, a finding that is similar to the previously reported average number of RS-specific bands in 36 normal isolates of *B. japonicum* obtained from the Nakazawa field site (RSα, 9.4; RSβ, 7.6) (22). Strain USDA123, a prevalent *B. japonicum* serotype strain indigenous to the United States, was shown to have copy numbers of RS elements comparable to the Tokachi-type HRS isolates (Table 1), although strains USDA110 and USDA122 fell into the category of normal isolates.

Shift and reiteration of *nifDK*- and *hupLS*-specific hybridization profiles. In general, DNA sequences in and around *nifDK* and *hupLS* genes of *B. japonicum* isolated from soybeans in fields are well conserved (21, 22). Hybridization specific for *nifDK* with *Hin*dIII-digested total DNAs from normal isolates from a field site at Ami consistently showed a 9.5-kb hybridization band (Fig. 1A). However, when *B. japonicum* field isolates from the Nagakura field site containing many HRS isolates were tested, we observed variation among the *nifDK*-specific hybridization bands in HRS isolates (Fig. 1A). Moreover, we observed reiteration of *hupLS*- specific bands when *Hin*dIII-digested total DNA of some isolates from the Nagakura field were hybridized (Fig. 1B). These results suggested that genomic rearrangements involving RS around the *nif* and *hup* genes of HRS isolates may have given rise to these band differences and reiterations.

Nucleotide sequences of 16S rRNA gene. To examine whether HRS isolates belong to a different genus or species from *B. japonicum*, we analyzed nucleotide sequences (ca. 1 kb) of the 16S rRNA genes of HRS isolates NK5 and NK6 and normal isolate NK2 and compared these with the corresponding published sequences of *B. japonicum* and its neighbors. Figure 2 shows a phylogenetic tree. Two HRS isolates, NK5 and NK6, and normal isolate NK2 could be grouped into a *B. japonicum* cluster that included USDA110 (L2330 and Z35330), indicating that these HRS isolates also belong to *B. japonicum* in terms of phylogeny based on 16S rRNA gene sequences.

Symbiotic phenotypes and growth rate. Symbiotic phenotypes showed no difference between the HRS and normal

TABLE 1. Estimated copy number of RSα and RSβ and the mean generation time in *B. japonicum* HRS and normal isolates

Isolate or strain	Estimated copy number[a]		Mean generation time (h)
	RSα	RSβ	
Normal isolates			
NC4a	8	9	5.8
NC6a	9	6	6.9
NC41a	7	7	5.8
NK2	7	8	6.6
NK8	9	9	6.0
NK23	7	7	6.6
T7	5	3	7.6
T9	7	2	5.9
T12	7	5	7.2
Mean ± SD	7.3 ± 1.2	6.2 ± 2.5	6.5 ± 0.7
Niigata-type HRS isolates			
NC3a	175	25	8.9
NC32a	131	34	10.1
NK4	144	38	10.3
NK5	171	45	11.2
NK6	118	22	9.9
NK9	115	23	10.0
NK10	132	38	10.8
NK16	99	23	11.2
NK18	106	24	9.5
NK20	150	29	10.4
NK25	105	29	10.5
NK28b	138	43	10.6
NK29	124	44	10.8
NK34	115	40	11.8
NK37	86	27	9.1
NK40	133	45	9.6
Mean ± SD	128 ± 25	33 ± 9	10.3 ± 0.8
Tokachi-type HRS isolates			
T2	19	41	8.3
T15	22	46	10.1
T22	17	51	13.1
T25	22	47	10.1
T31	23	35	8.6
Mean ± SD	21 ± 3	44 ± 6	10.0 ± 1.9
USDA reference strains			
USDA 110	12	6	6.2
USDA 122	8	7	6.5
USDA 123[b]	23	42	8.5

[a] Estimated copy numbers of HRS and normal isolates were determined based on the relative intensity and band number of RS-specific hybridization. To estimate the copy number of RSα and RSβ of HRS isolates and USDA 123, radioactivity was measured as described in the text. The band numbers of RS-specific hybridization of normal isolates and USDA 122 were counted.

[b] Strain USDA 123 had copy numbers of RSα and RSβ comparable to those of the Tokachi-type HRS isolates.

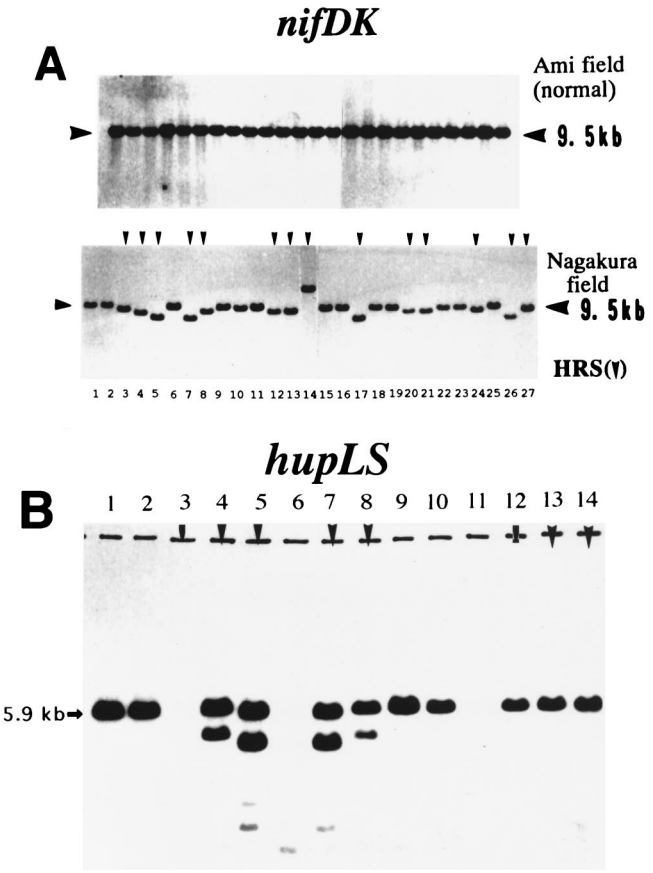

FIG. 1. Autoradiogram showing Southern blot hybridization of HRS and normal isolates of *B. japonicum* with *nifDK* (A) and *hupLS* (B). The arrowhead in the lanes and an asterisk in the designations below indicate an HRS isolate. (A) All isolates from the Ami field showed a consistent 9.5-kb band that hybridized with *nifDK* (top panel), whereas HRS isolates from the Nagakura field showed a shift of *nifDK*-specific hybridization bands below and above the 9.5-kb band (arrowheads, bottom panel). Each lane contained *Hin*dIII-digested total DNA from the following isolates: USDA110 (lane 1), NK2 (lane 2), NK4* (lane 3), NK5* (lane 4), NK6* (lane 5), NK8* (lane 6), NK9* (lane 7), NK10* (lane 8), NK13 (lane 9), NK14 (lane 10), NK15 (lane 11), NK16* (lane 12), NK18* (lane 13), NK20* (lane 14), NK21 (lane 15), NK23 (lane 16), NK25* (lane 17), NK26 (lane 18), NK28a (lane 19), NK28b* (lane 20), NK29* (lane 21), NK30 (lane 22), NK32 (lane 23), NK34* (lane 24), NK35 (lane 25), NK37* (lane 26), and NK40* (lane 27). (B) The *Hin*dIII-digested DNA of HRS isolates from the Nagakura field showed a duplex of bands of *hupLS*-specific hybridization. Isolates corresponding to lane numbers are the same as in panel A. NK5* (lane 4) and NK10* (lane 8) showed 5.8- and 4.9-kb bands of *hupLS*-specific hybridization. NK6* (lane 5) and NK9* (lane 7) showed 5.7- and 4.6-kb bands.

isolates with respect to nodulation, nitrogen fixation, and hydrogen uptake (data not shown). However, the growth rates of HRS isolates appeared to be slower than those of normal isolates. Thus, we compared the mean generation time between HRS and normal isolates (Table 1). The data indicated that the generation time of HRS isolates was longer than that of normal isolates. In particular, the Niigata-type HRS isolates, which possessed extremely high copy numbers of RSα (Table 1), showed significantly longer generation times than the normal isolates and the normal strains USDA110 and USDA122. The generation time of normal isolates was similar to the values (≥6 h) reported previously (34).

Comparison of serogroup and hydrogenase phenotype. To investigate the origin of the *B. japonicum* HRS isolates, we compared serogroups and hydrogenase types between normal and HRS isolates from three field sites (Fig. 3). The isolates tested did not react with antisera 76 and 94 of *B. elkanii* but did react with antisera of *B. japonicum* strains USDA110, USDA122, USDA123, USDA129, and J5033. Although double and triple cross-reactions were observed, the strains were classified into six serogroups. Groupings based on a combination of serogroup and hydrogenase phenotype of both HRS and normal isolates appeared to be related to the field sites. Serogroups 110 and Hup^+ were dominant in both HRS and normal isolates from the Nakazawa and Nagakura field sites, whereas serogroups 123-J5033 and Hup^- were dominant in both HRS and normal isolates from the Tokachi field site. These results

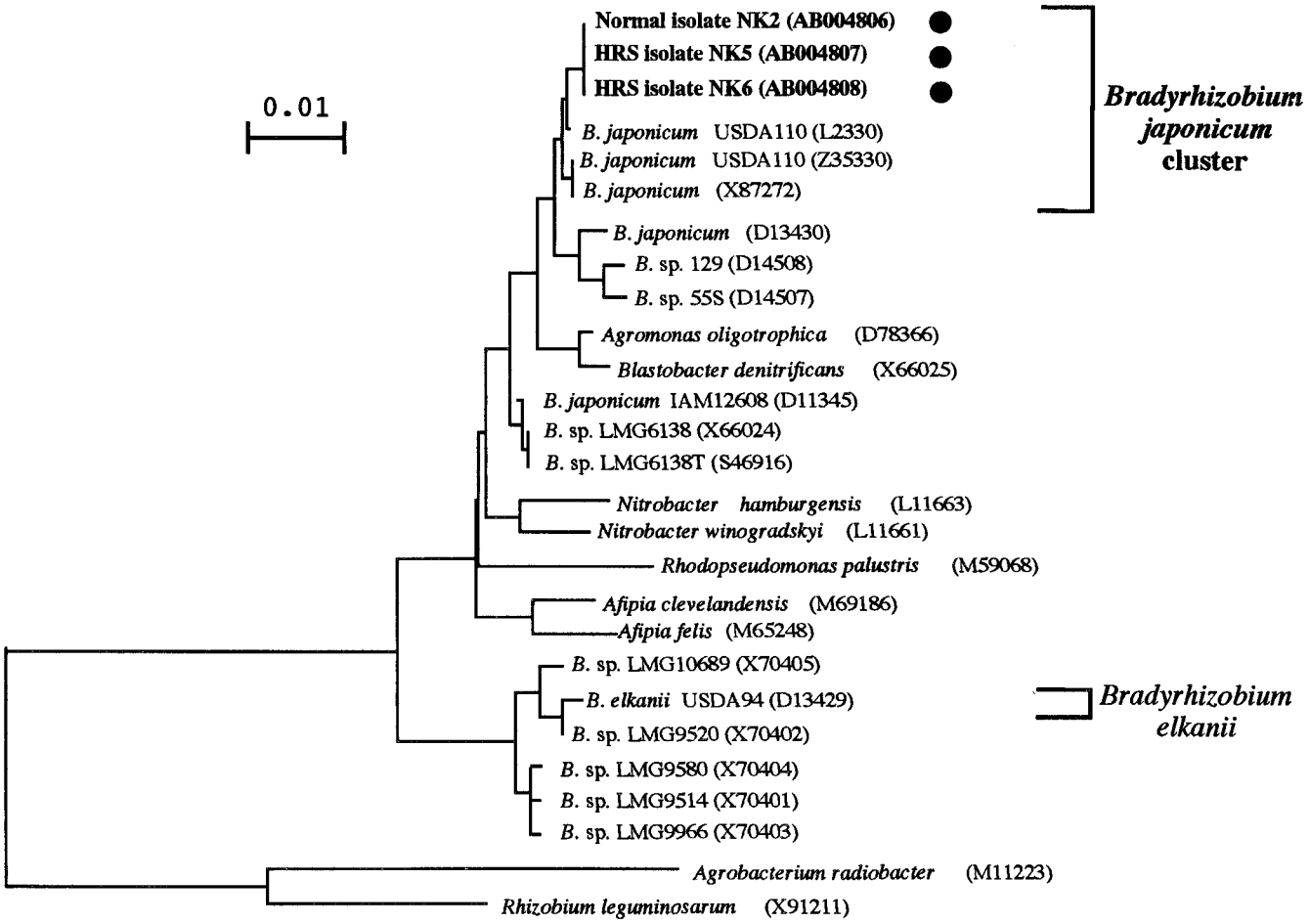

FIG. 2. Phylogenetic tree showing relatedness of HRS isolates by neighbor-joining grouping of the aligned sequences of the 16S rRNA gene (28). Black circles indicate HRS isolates and normal isolate from Nagakura field site. Bar indicates 0.01 base substitution per nucleotide.

suggested that HRS isolates are derived from indigenous normal isolates or vice versa at the local site.

RSα tandem repeat. In recent years, it has been reported that tandem repeats of the IS*30* element can give rise to a burst of DNA rearrangements, including site-specific deletions, inversions, and intermolecular transposition in *E. coli* (1, 24). The fact that Niigata-type HRS isolates possessed a high copy number of RSα prompted us to examine whether HRS isolates have RSα repeated sequences in tandem. Primers 13 and 14 were designed to detect close or contiguous tandem repeats of RSα sequences. When the structure exists, the expected size of the amplification product was 600 bp. PCR analysis with primers 13 and 14 showed that a 600-bp DNA fragment was amplified in Niigata-type HRS isolates as expected, whereas there was no amplification product in normal isolates from the Nakazawa and Nagakura field sites (Fig. 4). In Tokachi-type HRS isolates, which possessed a relatively low copy number of RSα, the 600-bp DNA fragment was observed only in HRS isolate T2 (Fig. 4). To precisely determine the sequence at the junction of the RSα tandem repeats, PCR amplification was performed by using primers 17 and 18 and the 600-bp products of HRS isolates NC32a, NK5, and T2 as templates. The amplification products (ca. 240 bp) were then cloned and sequenced. The resultant DNA sequences were aligned with RSα7 (17) (Fig. 5). In these isolates, RSα sequences were formed which were tandemly repeated with four nucleotides (5′-CTAG) shared between them (Fig. 5A). In isolate NK5, a second pair of tandem RSα repeats was found with a spacer region of 35 nucleotides between them (Fig. 5B). The fact that two RSα units shared the four nucleotides (5′-CTAG) in the tandem repeat structure suggested that RSα might target to the four nucleotides (5′-CTAG) of the original RSα, when the new RSα is transposed in HRS isolates (Fig. 5A). The 4-bp sequence is therefore likely to be a target site in RSα transposition.

DISCUSSION

Niigata-type HRS isolates are unique in that they have a tandem repeat RSα dimer and an extremely high RSα copy number, abnormal patterns of hybridization with *nif* and *hup* genes, a longer generation time, and a similar serotype grouping (serogroups 110 and 122). To our knowledge, this is the first report describing the characteristics of Niigata-type HRS isolates of *B. japonicum*. On the other hand, Tokachi-type HRS isolates appear to be found in the United States because strain USDA123 belonged to the group of HRS isolates of this type in terms of the estimated copy number of RSα and RSβ. Rodriguez-Quinones et al. (27) found hyper-reiterated DNA regions that are conserved among *B. japonicum* serocluster 123 strains, and they subsequently determined the DNA region,

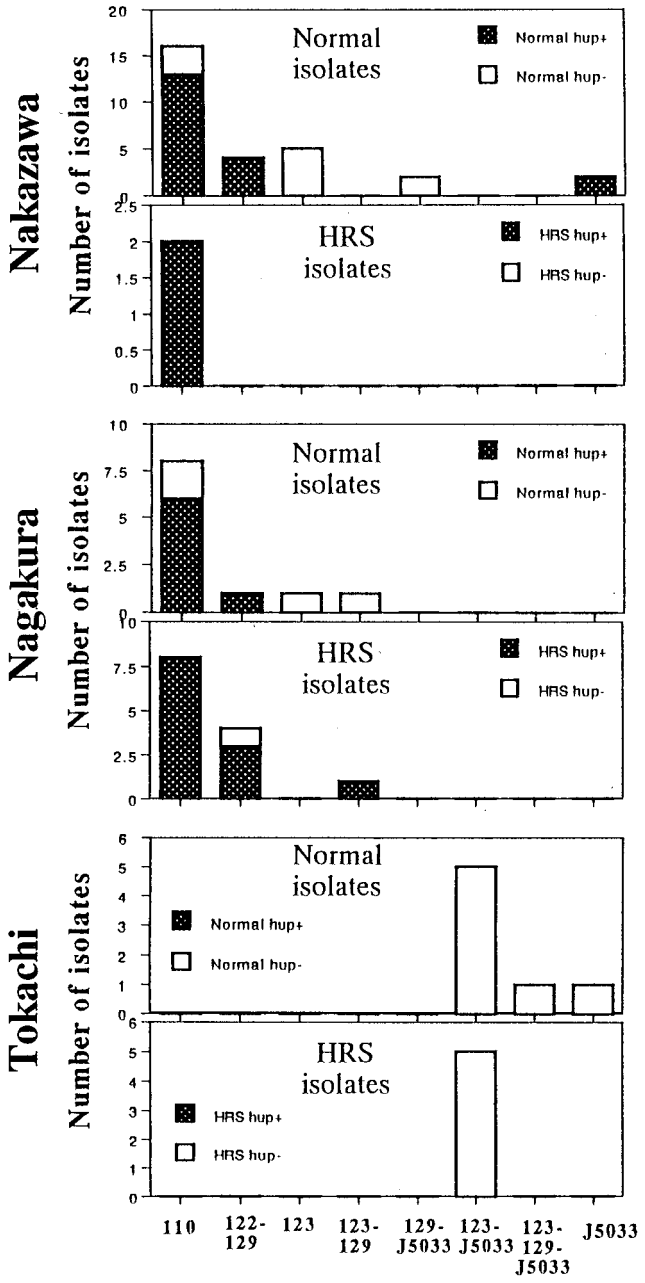

FIG. 3. Comparison of serogroup and *hup* phenotype between normal and HRS isolates of *B. japonicum* from three field sites. The field isolates tested were as follows: the isolates from Nagakura were NK2, NK8, NK13, NK14, NK15, NK21, NK23, NK26, NK28a, NK32, NK35, NK4*, NK5*, NK6*, NK9*, NK10*, NK16*, NK18*, NK29*, NK25*, NK28b*, NK34*, NK37*, and NK40*, and the isolates from Tokachi were T7, T8, T9, T10a, T10b, T12, T29, T39, T40, T2*, T15*, T22*, T25*, and T31*, where an asterisk indicates an HRS isolate. Isolates NC4a NC6a, NC41a, NC3a*, and NC32a* from the Nakazawa field, which had not shown clear agglutination reactions previously, were tested again by using a relatively large amount of fresh cells. For other *B. japonicum* isolates from the Nakazawa field, previous data of serogroup and the Hup phenotype (22) were used.

HRS1, which has properties similar to those of an IS element (15) and RSα sequences.

The estimated copy numbers of RSα in the 16 Niigata-type HRS isolates ranged from 86 to 175, with a mean of 128 copies (Table 1). The RSα copy number in Niigata-type HRS isolates appeared to be significantly higher than normally found for IS

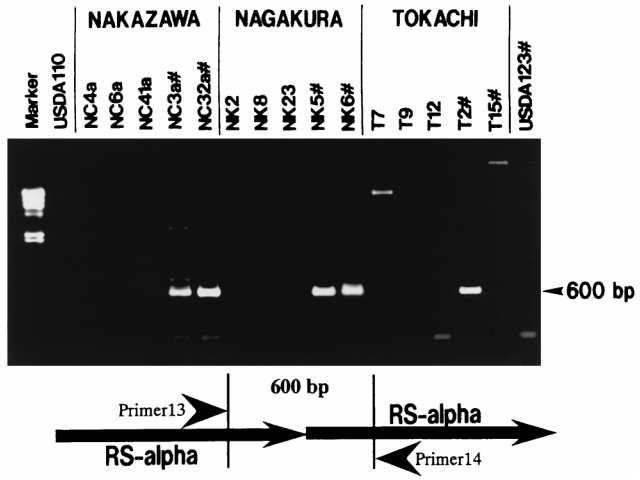

FIG. 4. Detection of a 600-bp PCR product consistent with the occurrence of a tandem repeat RSα dimer. #, HRS isolate.

elements in bacteria (7). Among natural isolates of *E. coli*, maximum and mean copy numbers of IS*1*, IS*2*, IS*3*, IS*4*, IS*5*, and IS*30* were fewer than 27 and 8, respectively (7, 30). Comparably low numbers are found in plant-associated bacteria such as *Rhizobium* (5, 35), *Agrobacterium* (5), and *Xanthomonas* (2) spp.

However, it has been known that *Shigella dysenteriae* (25), *Acetobacter pasteurianus* (16), and *Halobacterium* spp. (9, 29) possess high copy numbers of iso-insertion sequences of IS*1*, IS*1380*, and ISHs, respectively, which are comparable to the number of RSα copies found for the Niigata-type HRS isolates. The high copy numbers of these IS elements are associated with DNA rearrangements and genome instability in

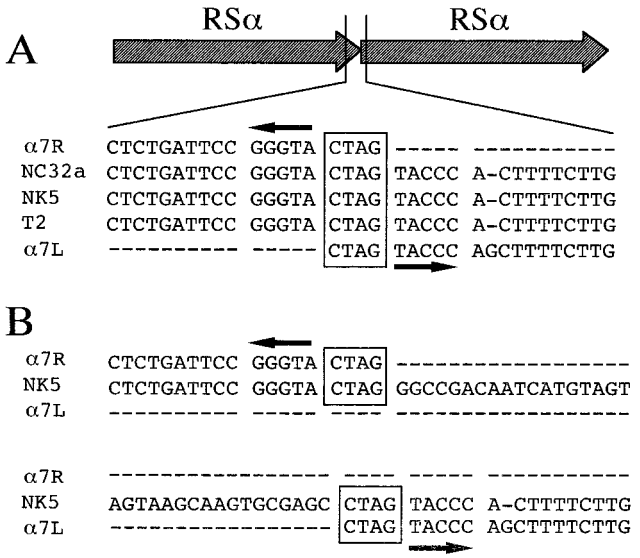

FIG. 5. Structure of a tandem repeat $(RS\alpha)_2$ and its nucleotide sequences at its junction in the HRS isolates NC32a, NK5, and T2. (A) The shortest and predominant PCR products obtained with the total DNAs of NC32a, NK5, and T2 as templates were cloned and sequenced. α7R and α7L are the right and left ends of the RSα7 sequence of *B. japonicum* USDA110 (17). (B) The nucleotide sequence of isolate NK5 at the junction of a tandem repeat $(RS\alpha)_2$ contains 35 nucleotides interrupting the two RSα sequences. The arrow indicates the terminal inverted repeat of RSα. Boxed sequences indicate a putative target duplicate.

these bacteria. In Niigata-type HRS isolates possessing a high copy number of RSα, the variation in *nifDK*-specific hybridization bands and the reiteration of *hupLS*-specific hybridization bands suggested that DNA rearrangements might occur in the symbiotic regions.

It has been reported that tandem repeats of IS elements separated by a few base pairs are active in the transposition of IS*21* (26), IS*3* (33), and IS*30* (1, 24). Since the tandem repeat RSα dimer $(RS\alpha)_2$ in HRS isolates is very similar to that of $(IS30)_2$, it is possible that this causes DNA rearrangements in HRS isolates.

In general, the number of copies of IS elements in the genome increases without causing deletions because bacterial IS elements transpose conservatively. The corollary is that the IS copy number should generally increase in the evolution of strains of bacteria (7). It may be that HRS isolates have evolved from normal isolates by transposition and recombination events by amplification during DNA replication of arrays of tandemly repeated sequences which include the IS elements. The resultant HRS isolates are considered to exhibit extra-slow growth. Groupings based upon serogroup and hydrogenase phenotype suggested that such events have independently occurred in individual different field ecosystems.

Gross et al. (8) and Xu et al. (36) described extra-slow-growing (ESG) soybean bradyrhizobia that are indigenous to alkaline and Chinese soils, respectively. Xu et al. (36) proposed the name *Bradyrhizobium liaoningens* sp. nov. for the ESG strains from China based on many taxonomic features. The HRS isolates appear to resemble ESG strains. Both nodulated soybeans grew slowly in a free-living state (Table 1) and were sensitive to antibiotics such as tetracycline (data not shown). However, it is clear from the sequences of the 16S rRNA genes of the HRS isolates and the strain *B. japonicum* USDA110 that they are closely related. Moreover, the serotypes of HRS isolates were within the range of *B. japonicum*. Therefore, HRS isolates should be classified as *B. japonicum*.

The reason the Niigata-type HRS isolates were isolated only at two field sites of the Niigata Agriculture Experiment Station is not clear. The presence of *B. japonicum* HRS isolates in nature suggests that they might give rise to genetic diversification and adaptation of the bacteria to specific environments and so play an important role in the evolution of symbiotic bacteria including gene transfer. Further studies are necessary to understand the significance of *B. japonicum* HRS isolates in field populations.

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